Abstract. – This article explores current evidence on the role of oxidative stress in viral infections, and on the use of antioxidant drugs as adjunctive treatment. MEDLINE/PubMed was searched for appropriate keywords, and preclinical and clinical studies with reviews were retrieved and examined by authors.

Old and current evidence shows that GSH content reduction is the main mechanism of redox imbalance in viral-infected cells. Clinical studies found that GSH levels are depleted in patients with viral infections such as HIV and SARS-CoV. Viral infections activate inflammation through different pathways, and several of these mechanisms are related to oxidative stress. NAC is a precursor of GSH, and many of its intracellular effects are mediated by GSH replenishment, but it also activates some anti-inflammatory mechanisms. NAC has an excellent safety profile and better oral and topical bioavailability than GSH. These characteristics make NAC a suitable option as a repurposed drug.

Adjunctive antioxidant treatment may improve the outcomes of antiviral therapies. Current evidence supports the rationale for this practice and some clinical experience showed encouraging results.

Key Words: Oxidative stress, Cytokines, Glutathione, N-acetylcysteine, Viral infection.

Introduction

The recent COVID-19 pandemic has prompted research on treatments for managing viral respiratory infections and has drawn attention to drugs suitable for repurposing and likely viable for rapid introduction as adjunctive therapy. This line of research has drawn information from recent preclinical and clinical experience on SARS-CoV-2 and previous observations on other viruses.

Oxidative stress and increased release of cytokines have been among the main mechanisms responsible for infection and disease. They have been suggested as effective therapeutic targets for respiratory viral infections. The effects of antioxidants were investigated, and N-acetylcysteine (NAC), which was found to inhibit viral replication several years before, was presented as a candidate for repurposing, being a safe and experienced drug with antioxidant and anti-inflammatory activities.

Methodology

This review explores current evidence on the role of oxidative stress in viral infections. It discusses the antioxidant mechanisms involved, with the clinical perspectives for the therapeutic use of antioxidant drugs in the adjunctive treatment of viral infections.

To address the objective of this article, a review of the literature has been carried out. MEDLINE/PubMed was scoured for appropriate keywords: “oxidative stress”, “inflammation”, “viral infection”, “SARS-CoV-2”, “HIV”, “influenza”, “NAC”, “glutathione (GSH)”, and “anti-oxidant”. In vitro and in vivo preclinical studies, clinical studies, reviews, and meta-analyses were retrieved; articles in English or English abstracts were considered. All retrieved articles were read and examined by authors and were selected based on relevance. This selection was based on the authors’ clinical and scientific expertise. A narrative review article was written, reporting published evidence and the expert opinion of the authors.
Oxidative Stress Role in Viral Infections

When respiratory viral infection occurs, blood and tissue redox markers are imbalanced in infected cells. In contrast, lung infection is associated with cytokine production, inflammation, cell death, and tissue damage, triggered by enhanced reactive oxygen species (ROS) production1,7.

Many years ago, HIV-positive patients were found to have reduced GSH and increased malondialdehyde and total hydroperoxide plasma levels. In these patients, lymphocytes showed increased DNA fragmentation and a significant reduction of glutathione peroxidase (GPx), and erythrocytes had an increased superoxide dismutase activity8. Other dated evidence on oxidative stress following viral infection demonstrated that Madin-Darby canine kidney cells infected with Sendai virus showed rapid loss of GSH without an increase in oxidized products. In this study, loss of GSH was mainly due to membrane damage upon virus fusion9. Indeed, the GSH cell content reduction can result from decreased GSH synthesis or increased consumption, degradation or transport/leakage, or a combination of these factors, as found in HIV-infected cells2,10.

Such results have been confirmed and developed in the following years, and it is now known that GSH content reduction is the main mechanism of redox imbalance in viral-infected cells11. Indeed, GSH is an important nucleophilic scavenger and an enzyme-catalyzed antioxidant, with a major role in protecting from oxidative tissue injury12.

The production of ROS and reactive nitrogen species (RNS) is increased in moderate and severe septic shock due to many viral diseases such as SARS-CoV13,14. This redox imbalance is associated with elevated expression of iNOS, NADP oxidases, COX-2, and xanthine oxidase, which activate transcription factors, NF-kB; an inflammatory response results, and its progression ensues13,14.

It has been found that GSH levels are depleted in plasma, epithelial lining fluid, peripheral blood mononuclear cells, and monocytes in asymptomatic HIV-infected subjects and AIDS patients15. Moreover, clinical studies have shown that GSH deficiency is correlated with morbidity of AIDS16. GSH deficiency contributes to the induction of apoptosis in CD4+ T lymphocytes8,10. Decreased GSH levels have been shown to allow activation of NF-kB, with downstream events facilitating HIV expression, and this pathway was blocked by NAC supplementation17.

Whereas GSH depletion has been associated with viral infections, an increase in GSH intracellular levels has been demonstrated to inhibit HIV replication by blocking NF-kB activation due to oxidative stress and interfering with HIV entry through induction of redox changes in the CD4 D2 domain18. Moreover, high levels of GSH inhibit the correct folding and stabilization of viral protein, thus preventing the production of infectious virus particles5. Finally, the virion-associated reverse transcriptase, which plays a crucial role in HIV replication, is inhibited in the presence of GSH19.

In addition, studies on the influenza virus have demonstrated a facilitating role in oxidative stress. Briefly, GSH levels decrease during virus replication, as GSH modulates the life cycle of the influenza virus. This modulation is linked with the antiapoptotic protein Bcl-2, contributing to enhancing intracellular GSH concentrations. Finally, GSH inhibits influenza virus replication20.

Progressive depletion of GSH with an oxidative stress state was observed during parainfluenza-1 Sendai virus infection, and the administration of exogenous GSH significantly inhibited virus replication21. Rhinovirus upregulates its cellular receptor, ICAM-1, in respiratory epithelial cells, and this mechanism depends on the activation of an NF-kB-binding element in the ICAM-1 promoter. GSH can significantly inhibit rhinovirus-induced ICAM-1 upregulation via inhibition of NF-kB activation22,23.

During the recent pandemic, hospitalized patients with COVID-19 presented severe GSH deficiency, increased levels of thiobarbituric acid reactive substances expressing oxidative stress, and F2-isoprostane, indicative of oxidant damage, compared to uninfected controls. This oxidant damage was present at any age but worsened with increasing age24.

Inflammation in Viral Infections

Inflammatory mechanisms have a major role in the pathophysiology of viral infections, as many pathological findings may suggest. For example, the pathology of ARDS in infections with SARS coronavirus or H5N1 avian influenza virus is characterized by an accumulation of inflammatory cells, edema, and a relevant increase in cytokines25,26. Patients with COVID-19 have a deregulated immune system with decreased lymphocyte count, elevated leukocyte count and neutrophil-lymphocyte-ratio,
and reduced percentages of basophils, eosinophils, and monocytes. Levels of inflammatory cytokines are high, mainly in severe cases, and infection-related biomarkers are present.

Viral infections activate inflammation through different pathways, and several of these mechanisms are related to oxidative stress. Proinflammatory cytokines are released by cells exposed to ROS, and, conversely, lipopolysaccharide (LPS) induces both intracellular accumulation of ROS and release of IL-1β, IL-6 and TNF-α. Inflammatory cytokines are released through a redox-dependent pathway independent from NF-κB activation and enhanced by GSH depletion.

NF-κB-dependent cytokine release is also relevant, as suggested by resistance to acid-induced acute lung injury (ALI) of mice mutant for Toll-like receptor 4 (TLR4), which is necessary for NF-κB activation. The TLR4 Toll/IL-1 receptor domain-containing adaptor inducing the IFN-β (TRIF) pathway is involved in inducing cytokine production by macrophages and lung injury caused by oxidized phospholipids. These phospholipids are produced in the lungs of humans and animals infected with SARS, anthrax or H5N1.

The contribution of TLR4 to the pathogenesis of COVID-19 is relevant, as its activation causes an excessive innate immune response. SARS-CoV-2 binds TLR4 and activates TLR4 signaling resulting in increased cell surface expression of ACE2, the binding site of SARS-CoV-2, and facilitation of virus entry. Furthermore, SARS-CoV-2 destroys the type II alveolar cells that secrete pulmonary surfactants, which normally block TLR4 in the lungs by decreasing the air/tissue surface tension; thus, alveolar cell destruction promotes ARDS and inflammation.

Complement may be involved in the pathogenesis of coronavirus infection disease. Activation of complement (sc5b-9 and C5a) was found in patients with COVID-19, with significantly higher plasma levels in the patients with severe disease than in those with moderate disease. These findings agree with experimental data showing that C3 knockout mice infected with SARS-CoV have less lung disease than wild-type mice.

**N-Acetylcysteine**

NAC is a precursor of GSH, and many of its intracellular effects are mediated by GSH replenishment. At the intracellular level, NAC is deacetylated to yield L-cysteine (L-cys), which is the rate-limiting amino acid in GSH synthesis. Increased availability of L-cys promotes the production of the rate-limiting enzyme glutamate-cysteine ligase (GCL), which may be activated to synthesize GSH.

NAC has long been used in clinical practice, since the 1960s, as a drug with mucolytic and antioxidant activity, for respiratory diseases and the treatment of acetaminophen poisoning. It has an excellent safety profile and better oral and topical bioavailability than GSH. Its ability to break the disulfide bonds is responsible for the depolymerization of mucin and mucolytic. Breaking disulfide bonds may also reduce the affinity of SARS-CoV-2 for the ACE2 sites, thereby reducing the entry of the viruses into the cell. Intravenous NAC at doses as high as 150 mg/kg is standard practice as an approved antidote against acetaminophen intoxication, with efficacy up to 100%, if administered within 8 hours from ingestion.

Indeed, the replenishment of GSH in cells activates many mechanisms. Sulphydryl groups (-SH) of GSH react with electrophilic metabolites in the cell nucleus, resulting in the binding of reactive DNA metabolites and blocking reactive intermediates. Besides its intracellular antioxidant activity, mainly mediated by GSH replenishment, NAC is able to effectively regenerate the free form of Cys34 of human serum albumin, which represents the major and predominant extracellular antioxidant, by breaking the disulfide bond of the cysteinylated form of albumin in plasma.

Antioxidant activity is also exerted by inducing p53-mediated apoptosis. Through the generation of L-cys, NAC acts as a hydrogen sulfide donor, which is a readily diffusible vasodilator and anti-inflammatory molecule.

A relevant mechanism in the antioxidant activity of NAC is due to the scavenging of ROS and especially of hypochlorous acid (HOCl) and •OH through the SH-groups. NAC molecules can also scavenge some RNS responsible for the oxidation of lipids, proteins, and DNA. NAC inhibits the previously mentioned oxidative stress-mediated activation of NF-κB and the dependent pathways for the upregulation of proinflammatory cytokines.

NAC activates several anti-inflammatory mechanisms by inhibiting oxidative stress or acting on inflammation mediators. NAC downregulates inflammation and favors transcription of phase II enzyme genes, reducing the stimulation of Nrf2, a high sensitivity transcription factor involved in the cellular antioxidant response.

NAC was found to elicit an anti-inflammatory activity secondary to neurokinin A (NKA) reducing...
tion and secondarily of IL-6, thus modulating a vicious circle between oxidative stress and neurogenic inflammation. By inhibiting EGFR, a tyrosine kinase involved in inflammation, α1-antitrypsin inactivation is decreased, thereby improving cell protection from inflammatory cell enzymes. In addition, via a GSH-mediated mechanism, NAC improves the structural conformational integrity of α1-antitrypsin and enhances α1-antitrypsin transcytosis, thus improving its cellular uptake and functions.

The mechanisms induced by NAC of decreased levels of IL-8, IL-6, ICAM, and the soluble alpha receptor for tumor necrosis p55 could be attributed to controlling the inflammatory immune response. NAC was demonstrated to inhibit the stress-induced expression of mucins. NAC has been demonstrated to exert also an H$_2$S-generating effect. NAC can be desulfurated to H$_2$S, a gasotransmitter protective against oxidative stress, which in turn may be oxidized within mitochondria to generate sulfane sulfur species. It was hypothesized that H$_2$S might counteract SARS-CoV-2 through several targets: host receptors for viral entry, RNA-dependent RNA polymerase necessary for viral replication, and the TLR4 pathway and NLRP3 inflammasome, which plays an important role in the COVID-19 cytokine storm. The main mechanisms activated by NAC in viral infections are depicted in Figure 1.

**Preclinical Studies of Anti-Oxidants Activity in Viral Infections:**

**Focus on NAC**

Several preclinical studies suggested using NAC as an antioxidant in experimental viral infections. The mechanisms of action of NAC on inflammation and oxidative stress have been demonstrated in *in vitro* and *in vivo* preclinical studies investigating possible targets for viral infection treatment.

Adding GSH in canine kidney cells or human small airway epithelial cells decreased the production of influenza virus particles upon infection. GSH inhibited the expression of viral matrix protein, caspase activation, and *Fas* upregulation. The same researchers found that administration of GSH with drinking water to BALB/c mice decreased the viral titer in the lung and trachea 4 days after intranasal inoculation of the mouse-adapted influenza A/X-31 strain.

NAC inhibited the replication of the H5N1 influenza A virus and proinflammatory molecule expression in adenocarcinoma human alveolar basal epithelial (A549) cells. Zhang et al demonstrated decreased production of proinflammatory molecules by NAC. In the lungs of BALB/c mice inoculated intranasally with A/swine/HeBei/012/2008/ H9N2 influenza virus, TLR4 protein and mRNA levels were reduced. Additionally, NAC inhibited pulmonary inflammation and edema as well as myeloperoxidase (MPO) activity, total cells, neutrophils, macrophages, TNF-α, IL-6, IL-1β, and chemokine (C-X-C motif) ligand-10 (CXCL-10) in the bronchoalveolar lavage fluid. NAC inhibited the overexpression and increased release of MUC5AC, IL-8, IL-6, and TNF-α induced by influenza viruses A and B and with a respiratory syncytial virus in alveolar type II epithelial cells.

Some studies suggested a possible advantage of combining NAC with a different treatment. The administration of a suboptimal dose of NAC slightly impacted the survival in a murine model of lethal influenza infection. Similarly, the administration of oseltamivir partially improved the survival of infected mice. On the contrary, the combination of NAC and oseltamivir increased survival to 100%. Similar results were obtained in mice infected intranasally with a lethal dose of influenza A virus APR/8 and treated with NAC alone or ribavirin alone, which were slightly effective, or with the combinations of the two drugs increasing the 14-day survival to 92%.

**The Role of Pollution**

The role of oxidative stress in viral infection pathophysiology has been confirmed by studies showing that air pollutants reduce host defenses, resulting in decreased resistance to respiratory infections. For example, exposure of mice to diesel exhaust during infection with influenza virus was associated with an increase in viral titers on days 4 and 8 post-infection. The bronchoalveolar lavage showed increased neutrophils and protein content, and IL-4 expression and production were significantly increased on days 1 and 4, while expression of the Th1 cytokines, IFN-γ, and IL-12p40 was decreased. Treatment with NAC prevented changes in cytokine expression and levels and lung inflammation, although diesel-enhanced virus titers were not changed. Interestingly, treatment with high-dose NAC for 6 months was demonstrated to reduce exacerbation incidence and to improve symptoms and quality of life in patients with COPD.
Inhibition of Viral Infection and Replication by NAC

Binding of the receptor-binding domain (RBD) to ACE2 is necessary for SARS-CoV-2 binding to the cell surface, and the Cys-488 in the RBD spike is required for SARS-CoV-2 spike functions and infectivity. NAC inhibits the virus entry through this pathway, which may represent a target for early COVID-19 therapy\(^{31}\). Molecular dynamic simulations showed that the binding affinity of SARS-CoV-2 to ACE2 was significantly impaired when all the disulfide bonds of both ACE2 and SARS-CoV/CoV-2 spike proteins were reduced to thiol groups, adding to molecular bases for the severity of COVID-19 infection due to oxidative stress\(^{54}\).

The antioxidant activity of GSH interferes with the late stages of HIV replication in chronically infected macrophages by inhibiting HIV envelope glycoproteins (gp120), a known reservoir of the virus in the body\(^{5}\). Both GSH and NAC inhibited the induction of HIV-1 expression in a chronically infected promonocytic cell line (U1/HIV) and human primary cultured monocyte/macrophages. The two compounds decreased HIV-1 p24 antigen levels and the reverse transcriptase activity\(^{5}\).

Clinical Experiences with NAC in Viral Infections

Although large clinical trials are not available, several clinical experiences confirm the evidence from preclinical studies and suggest a role for NAC in treating viral infections. The administration of GSH concomitantly with standard treatment relieved the dyspnea associated with COVID-19 pneumonia in two patients\(^{55}\). In patients with septic shock, a shorter mechanical ventilation time and fewer days of ICU stay were necessary upon treatment with NAC\(^{43}\). The administration of...
NAC (2 × 600 mg/day for 8 weeks) to peritoneal dialysis patients in a placebo-controlled study reduced plasma levels of inflammatory markers, including complement (C3)36,57. In humans, during influenza infection, NAC inhibited the induction of proinflammatory cytokines through endosomal TLR3/hemagglutinin (HA)-induced ROS-dependent NF-κB activation58.

A total of 262 subjects (78% were aged ≥65 years and 62% suffered from non-respiratory chronic-degenerative diseases) were randomized to receive either placebo or oral NAC 600 mg twice daily for 6 months. The group receiving NAC experienced a significantly decreased frequency of influenza-like episodes, severity, and length of time confined to bed. Subjects with influenza syndrome had reduced symptoms in the NAC group. Nevertheless, the frequency of seroconversion towards the A/H1N1 Singapore 6/86 influenza virus was similar in the two groups. Only 25% of the virus-infected subjects under NAC treatment developed a symptomatic form, versus 79% in the placebo group59.

Recently, the addition of NAC solution for inhalation to oseltamivir for the treatment of viral infection was associated with a significantly shorter time to the resolution of cough, sore throat, fever, cold sweat and fatigue compared to patients receiving oseltamivir alone (p<0.05). Moreover, the levels of inflammatory factor lymphocyte ratio (LY), C-reactive protein (CRP) and procalcitonin (PCT) were lower in patients treated with NAC (p<0.05)60.

**Experiences in COVID-19**

A retrospective study compared standard of care alone versus standard of care and additional NAC 600 mg bid orally for 14 days, in patients hospitalized with moderate or severe COVID-19 pneumonia. Compared to the control group, treatment with oral NAC led to significantly lower rates of progression to severe respiratory failure (p<0.01), significantly lower 14- and 28-day mortality (p<0.001 and p<0.01 respectively), and progressively improved PO2/FiO2 ratio and decreased white blood cell, CRP, D-dimers and LDH levels61.

Another retrospective study on 1,083 patients hospitalized for COVID-19 pneumonia found that hospital stay was shorter in patients receiving NAC administered at a dosage of 300 mg intravenous TID, switched to 600 mg per os BID once it reached clinical stability and continued until discharge. However, no impact of NAC on short- and long-term outcomes, including in-hospital mortality, ICU admission, impairment of lung diffusing capacity for carbon monoxide, and chest X-ray alterations at the 6-month follow-up, was present62.

The use of oral NAC at doses of 600 mg every 8 hours in addition to other treatments was associated with significantly lower mortality (OR 0.56; 95% CI: 0.47-0.67) in a community-based study of 19,208 patients hospitalized with a diagnosis of COVID-19, despite these patients being older, more frequently male and with more comorbidities. On the contrary, there were no significant differences with the use of NAC on the mean duration of hospitalization, admission to the intensive care unit or use of invasive mechanical ventilation63.

Significantly improved respiratory parameters with a shorter hospital stay were found in a case-control study, including 46 patients with confirmed COVID-19 receiving NAC therapy at a daily dose of 1200-1800 mg intravenously within 10 days (p=0.01)64.

**Conclusions**

Based on the evidence presented in this review, many scientific data underlined as oxidative stress could play a central role in promoting inflammation related to different diseases, including viral infections.

Viral infections are associated with increased production of ROS and decreased activity of antioxidant systems, with activation of a vicious circle where host defenses are reduced. The viral load is progressively increased, resulting in infectious disease. Several biological mechanisms are involved and may represent targets for therapeutic interventions.

We have reported different and interesting evidence supporting the possible use of an antioxidant as an add-on treatment during viral infections. Particularly, NAC could be used as an adjunctive treatment for respiratory viral infections. NAC is a safe drug with several action mechanisms and may be easily administered per os. Current evidence shows that NAC may interfere with virus link to cell receptors and with inflammation and oxidative stress induced by the infection. In addition, NAC may be protective against factors facilitating viral infections, such as pollution.

All these points allow the clinicians to have a drug that is easy to use, safe and well tolerated, opening an interesting perspective regarding the management of viral infections.

Thanks to the capacity of NAC to generate GSH in the lung by oral administration and the
possibility to modulate the dosage, this drug is a good option for clinical practice.

NAC is not an antiviral drug but could be used as an add-on treatment to obtain a good clinical answer thanks to a modulation of different biological systems that are correlated with oxidative stress during disease history and progression.

In conclusion, an adjunctive antioxidant treatment may improve the outcomes of antiviral therapies. Current evidence supports the rationale for this practice and some clinical experience showed encouraging results with the administration of NAC.

**Conflicts of Interest**

PS has received research grants from Air Liquide, Boehringer Ingelheim, Chiesi Farmaceutici, Pfizer, and fees for lectures, participation on advisory boards and consultancy from AstraZeneca, Berlin-Chemie, Zambon Italia, Zambon Brasil, Boehringer Ingelheim, Guidotti, Edmondpharma, Valeas, Sanofi. DR has received fees for participation on advisory boards and consultancy from AstraZeneca, Boehringer Ingelheim, Fondazione Internazionale Menarini, and GlaxoSmithKline, and fees for lecturing from AstraZeneca, Boehringer Ingelheim, Fondazione Internazionale Menarini. FD, AZ, SP, MS and AV have none to declare. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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**Authors’ Contributions**

Study conception and design: P. Santus; manuscript drafting: all authors equally contributed; approval to submit: all authors.

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